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Improvement of neuronal bioenergetics by neurosteroids: Implications for age-related neurodegenerative disorders

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ABSTRACT

The brain has high energy requirements to maintain neuronal activity. Consequently impaired mitochondrial function will lead to disease. Normal aging is associated with several alterations in neurosteroid production and secretion. Decreases in neurosteroid levels might contribute to brain aging and loss of important nervous functions, such as memory. Up to now, extensive studies only focused on estradiol as a promising neurosteroid compound that is able to ameliorate cellular bioenergetics, while the effects of other steroids on brain mitochondria are poorly understood or not investigated at all. Thus, we aimed to characterize the bioenergetic modulating profile of a panel of seven structurally diverse neurosteroids (progesterone, estradiol, estrone, testosterone, 3α-androstanediol, DHEA and allopregnanolone), known to be involved in brain function regulation. Of note, most of the steroids tested were able to improve bioenergetic activity in neuronal cells by increasing ATP levels, mitochondrial membrane potential and basal mitochondrial respiration. In parallel, they modulated redox homeostasis by increasing antioxidant activity, probably as a compensatory mechanism to a slight enhancement of ROS which might result from the rise in oxygen consumption. Thereby, neurosteroids appeared to act *via* their corresponding receptors and exhibited specific bioenergetic profiles. Taken together, our results indicate that the ability to boost mitochondria is not unique to estradiol, but seems to be a rather common mechanism of different steroids in the brain. Thus, neurosteroids may act upon neuronal bioenergetics in a delicate balance and an age-related steroid disturbance might be involved in mitochondrial dysfunction underlying neurodegenerative disorders.

1. Introduction

The brain is a highly differentiated organ with high energy requirements, mainly in the form of adenosine triphosphate (ATP) molecules. Despite its small size, it accounts for about 20% of the body's total basal oxygen consumption [1]. As a result, the brain is more sensitive to neuronal damage during hypometabolic states and impaired redox homeostasis, as observed in normal aging and in neurodegenerative diseases associated with a decline in energy production and changes in the redox status [2]. In this context, mitochondria, small organelles that are present in almost all cell types playing a predominant role in cellular bioenergetics, are particularly important in the nervous system because of its high energy demand. Mitochondria are not only the "powerhouses of the cell", providing the main source of cellular energy *via* ATP generation through oxidative phosphorylation, but they also contribute to plenty of cellular functions, including apoptosis, intracellular calcium homeostasis, alteration of the cellular reduction–oxidation (redox) state and synaptic plasticity [3,4]. Thus, it is more and more recognized that mitochondrial dysfunction is a significant and early event of neurodegeneration, and that the pathophysiological mechanisms of a range of neurodegenerative diseases, including Alzheimer's (AD) and Parkinson's disease (PD), are associated with a decline in bioenergetic activity and an increase in oxidative stress, particularly in mitochondria themselves [5–10].

Abbreviations: 3α-A, 3α-androstanediol; AD, Alzheimer's disease; APP, amyloid-β precursor protein; AP, allopregnanolone; D, DHEA (dihydroepiandrosterone); DHR, dihydrorhodamine 123; DMSO, dimethylsulfoxide; E1, estrone; E2, 17β-estradiol; E3, estriol; ECAR, extracellular acidification rate; ETC, electron transport chain; MAS, mitochondrial assay solution; MPP, mitochondrial membrane potential; mtROS, mitochondrial reactive oxygen species; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; P, progesterone; PD, Parkinson's disease; PMP, plasma membrane permeabilizer; RCR, respiratory control ratio; roGFP, redox sensitive green fluorescent protein; ROS, reactive oxygen species; SRA, steroid receptor antagonist; T, testosterone; TCA, tricyclic acid

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Steroid hormones are molecules involved in the control of many physiological processes in the periphery, from reproductive behavior to the stress response. They are mainly produced by endocrine glands, such as the adrenal glands, gonads and placenta, but in 1981 Baulieu and co-workers were the first to demonstrate the production of steroids within the nervous system itself [11]. This last category of molecules is now called "neurosteroids" and is defined as steroids that are synthetized within the nervous system independently of peripheral endocrine glands. Neurosteroid levels remain elevated even after adrenalectomy and castration [12,13] and are involved in brain-specific functions. Since the ability to produce neurosteroids is conserved during vertebrate evolution, one can suggest that this family of molecules is important for living things and that the modulation of their biosynthesis plays an important role in the pathophysiology of neurodegenerative disorders.

Studies performed in humans, animals, and cellular models have shown alterations in the synthesis of neurosteroids that declined during brain aging paralleled by a loss of important nervous functions, such as memory, and were further associated with PD and AD [14–16]. Thus, several neurosteroids have been quantified in various brain regions of aged AD patients and aged non-demented controls. This showed a general trend toward lower steroid levels in AD patients compared to controls, associated with a negative correlation between neurosteroid levels and amyloid-β (Aβ) and phospho-tau in some brain regions [17]. In accordance with these observations, previous data from our groups provided first evidence that, *vice versa*, Aβ and hyperphosphorylated tau differentially impacted neurosteroidogenesis (Fig. 1) [18–20]. Indeed, a decrease of progesterone and 17-hydroxyprogesterone production was observed in amyloid precursor protein (APP)/Aβ-overexpressing cells, while 3α-androstanediol and estradiol levels were increased [19]. Moreover, *in vitro* treatment of human neuroblastoma cells with "non-toxic" Aβ concentrations (within the nanomolar range) revealed an increase in estradiol production, whereas toxic Aβ concentrations (within the micromolar range) showed the opposite effect [18]. Overexpression of human wild type tau (hTau40) protein induced an increase in production of progesterone, 3α-androstanediol, and 17-hydroxyprogesterone, in contrast to the abnormally hyperphosphorylated tau bearing the P301L mutation that led to decreased production of these neurosteroids [19].

Moreover, a growing body of evidence has highlighted neuroprotective effects of steroids, particularly estradiol, against AD-related injury (reviewed in [21]). Because the drop of estrogen in a postmenopausal woman is considered as a risk factor in AD (two-thirds of AD patients are women), the neuroprotective action of estrogen has been widely investigated (reviewed in [22]). One *in vivo* study showed that estradiol treatment of ovariectomized female rats upregulated enzymes involved in glycolysis and oxidative phosphorylation, and increased ATP synthase expression which was translated into an increased mitochondrial respiration [23]. These findings were additionally confirmed in an AD mouse model by Yao and coworkers [24].

However, there is little evidence that other steroids are also able to act on mitochondrial function, and to our knowledge, no study has aimed to compare the effects of neurosteroids besides estradiol on cellular bioenergetics and redox environment in neuronal cells. Thus, the objective of our study was to investigate the effects of different neurosteroids on bioenergetic activity *in vitro*. For this purpose, we selected seven neurosteroids — progesterone, estradiol and estrone, belonging to the estrogen family; testosterone and 3α-androstanediol, belonging to the androgen family; and DHEA and allopregnanolone — known to be involved in brain function regulation [12,13,17–19,25]. Neurosteroid effects on ATP production, mitochondrial membrane potential (MMP), mitochondrial respiration, glycolysis and the consequences on the modulation of the redox environment were investigated in neuronal cells.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's-modified Eagle's medium (DMEM), RPMI-1640 medium, fetal calf serum (FCS), penicillin/streptomycin, progesterone, 17β-estradiol, estrone, 3α-androstanediol, DHR, TMRM, ADP, pyruvate, succinate and malate were from Sigma-Aldrich (St. Louis, MO, USA). Glutamax, MitoSOX, DPBS, neurobasal medium and B27 were from Gibco Invitrogen (Waltham, MA, USA). DHEA and allopregnanolone were from Calbiochem (Billerica, MA, USA). PMP and XF Cell Mitostress kit were from Seahorse Bioscience (North Billerica, MA, USA). Testosterone was from AppliChem (Darmstadt, Germany). Horse serum (HS) was from Amimed, Bioconcept (Allschwil, Switzerland). RU-486, ICI-187.780, and 2-hydroxy flutamide were from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Cell culture

Human SH-SY5Y neuroblastoma cells were grown at 37 °C in a humidified incubator chamber under an atmosphere of 7.5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 5% (v/v) heat-inactivated HS, 2 mM Glutamax and 1% (v/v) penicillin/streptomycin.

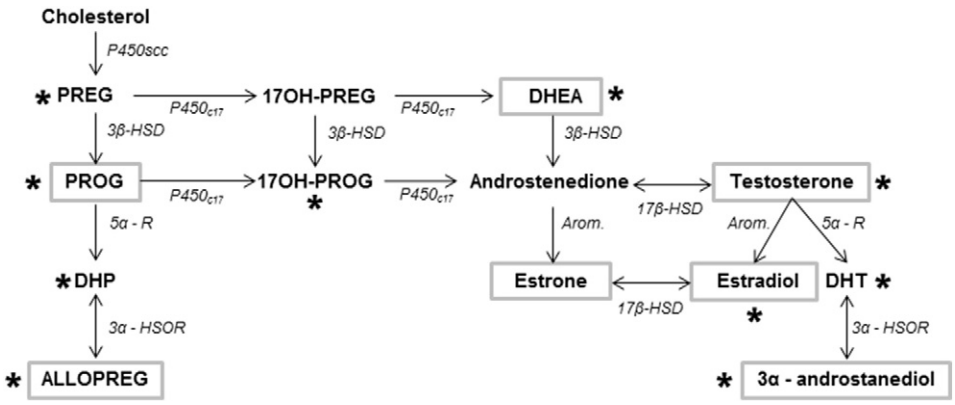

Fig. 1. Schematic representation of the main biochemical pathways for neurosteroidogenesis in the vertebrate brain. Boxes represent neurosteroids tested in our study. * indicates neurosteroids whose synthesis is impacted in AD. PREG; pregnenolone, PROG; progesterone, 17OH-PREG; 17-hydroxypregnenolone, 17OH-PROG; 17-hydroxyprogesterone, DHEA; dehydroepiandrosterone, DHP; dihydroprogesterone, ALLOPREG; allopregnanolone, DHT; dihydrotestosterone, P450scc; cytochrome P450 cholesterol side chain cleavage, P450c17; cytochrome P450c17, 3β-HSD; 3β-hydroxysteroid dehydrogenase, 5α-R; 5α-reductase, Arom.; aromatase, 21-OHase; 21-hydroxylase, 3α-HSOR; 3α-hydroxysteroid oxydoreductase, 17β-HSD; 17β-hydroxysteroid dehydrogenase.

Cells were passaged 1–2 times per week, and plated for treatment when they reached 80–90% confluence.

2.3. Primary neuronal cultures

Mouse cortical neurons were prepared from E15 embryos according to the French guidelines, as previously described [26]. Cells were plated in poly-L-lysine-coated plates at a density of 1.5×10^4 cells/well for ATP measurement (white 96-well plate) or 5×10^4 cells/well for measurement with the Seahorse XF24 Analyser (XF24 cell culture microplate). After 7 days at 37 °C, 50% of the medium was replaced with fresh medium every third day. ATP level, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were investigated in this primary neuronal culture after a 24 h treatment with the different neurosteroids.

2.4. Treatment paradigm

Assessment of cell viability was performed on SH-SY5Y neuroblastoma cells to determine the potential toxic concentration range of neurosteroids (from 10 nM to 1000 nM, data not shown) and steroid receptor antagonists (SRA, from 1 nM to 1 μM, data not shown) using a MTT reduction assay (Roche, Basel, Switzerland). On the basis of the MTT results, the concentrations 10 nM and 100 nM of steroids were then selected and used in all assays. SH-SY5Y cells were treated one day after plating either with DMEM (untreated control condition) or with a final concentration of 10 nM and 100 nM of progesterone, 17β-estradiol, estrone, testosterone, 3α-androstanediol, DHEA or allopregnanolone made from a stock solution in DMSO for 24 h (final concentration of DMSO < 0.002%, no effect of the vehicle solution (DMSO) alone compared to the untreated condition). In the experiment using SRA, cells were pre-treated for 1 h with 100 nM of RU-486 and ICI-187.780, and 1 μM of 2-hydroxy flutamide (2OH-flutamide), and then treated for 24 h with the corresponding neurosteroids. To limit cell growth and to optimize mitochondrial respiration, treatment medium contained only a low amount of fetal calf serum (5% FCS) as well as glucose (1 g/l) and was supplemented with 4 mM pyruvate. Each assay was repeated at least 3 times.

2.5. ATP levels

Total ATP content of SH-SY5Y cells was determined using a bioluminescence assay (ViaLighTM HT, Cambrex Bio Science, Walkersville, MD, USA) according to the instruction of the manufacturer, as previously described [27]. SH-SY5Y cells were plated in 5 replicates into a white 96-well cell culture plate at a density of 1.5×10^4 cells/well. The bioluminescent method measures the formation of light from ATP and luciferin by luciferase. The emitted light was linearly related to the ATP concentration and was measured using the multilabel plate reader VictorX5 (Perkin Elmer).

2.6. Cell proliferation assay

To verify if our treatment had an impact on cell cycle and induced proliferation, the BrdU Cell Proliferation Assay (Calbiochem, Darmstadt, Germany) was used following the instructions of the manufacturer. Briefly, SH-SY5Y cells were plated in 6 replicates into a 96-well cell culture plate at a density of 1×10^4 cells/well. During the final 12 h of neurosteroid treatment, BrdU was added to the wells and incorporated into the DNA of dividing cells. The detection of BrdU was performed using an anti-BrdU antibody recognized by a horseradish peroxidase-conjugated anti-mouse. After addition of the substrate (TMB), the color reaction was quantified using the multilabel plate reader VictorX5 at 450 nm. Values are proportional to the number of dividing cells.

2.7. Determination of mitochondrial membrane potential

The MMP was measured using the fluorescent dye tetramethylrhodamine, methyl ester, and perchlorate (TMRM). SH-SY5Y cells were plated in 6 replicates into a black 96-well cell culture plate at a density of 1.5×10^4 cells/well. Cells were loaded with the dye at a concentration of 0.4 μM for 15 min. After washing twice with HBSS, the fluorescence was detected using the multilabel plate reader VictorX5 (PerkinElmer) at 530 nm (excitation)/590 nm (emission). Transmembrane distribution of the dye was dependent on MMP.

2.8. Oxygen consumption rate and extracellular acidification rate

The Seahorse Bioscience XF24 Analyser was used to perform a simultaneous real-time measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). XF24 cell culture microplates (Seahorse Bioscience) were coated with 0.1% gelatine and SH-SY5Y cells were plated at a density of 2.5×10^4 cells/well in 100 μl of the treatment medium containing 5% FCS, 1 g/l glucose and 4 mM pyruvate. After neurosteroid treatment, cells were washed with PBS and incubated with 500 μl of assay medium (DMEM, without $NaHCO_3$, without phenol red, with 1 g/l glucose, 4 mM pyruvate, and 1% L-glutamine, pH 7.4) at 37 °C in a CO_2-free incubator for 1 h. The plate was placed in the XF24 Analyser and basal OCR and ECAR were recorded during 30 min. For primary neuronal culture, the same conditions were kept, except the medium; here DMEM was replaced by RPMI-1640 medium.

2.9. Mitochondrial respiration

The investigation of mitochondrial respiration was performed using the Seahorse Bioscience XF24 Analyser. XF24 cell culture microplates were coated with 0.1% gelatine and cells were plated at a density of 2.5×10^4 cells/well in 100 μl of treatment medium containing 5% FCS, 1 g/l glucose and 4 mM pyruvate. After neurosteroid treatment, cells were washed with 1× pre-warmed mitochondrial assay solution (MAS; 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO, 4.5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2 at 37 °C) and 500 μl of pre-warmed (37 °C) MAS containing 1 nM XF plasma membrane permeabilizer (PMP, Seahorse Bioscience), 10 mM pyruvate, 10 mM succinate and 2 mM malate was added to the wells. The PMP was used to permeabilize intact cells in culture, which circumvents the need for isolation of intact mitochondria and allows the investigation of the OCR under different respiratory states induced by the sequential injection of: i) ADP (4 mM) to induce state 3; ii) oligomycin (0.5 μM) to induce state 4o; iii) FCCP (2 μM) to induce state 3 uncoupled (3u); and iv) antimycin A/rotenone (0.5 μM and 1 μM respectively) to shut down mitochondrial respiration. Data were extracted from the Seahorse XF24 software and the respiratory control ratio (RCR: state 3/state 4o), which reflects the mitochondrial respiratory capacity, was calculated.

2.10. $GABA_A$ receptor expression

Cells were lysed and total RNA was extracted using the RNeasy Mini Kit from Qiagen (Venlo, Netherlands), according to the instructions of the manufacturer to measure $GABA_A$ receptor (subunits α1 and β2) mRNA levels. The first cDNA strand was synthesized using all RNA extracted by reverse transcription in a final volume of 30 μl using the Ready-to-Go You-Prime First-Strand Bead cDNA synthesis kit (GE Healthcare, Little Chalfont, UK) according to the supplied protocol. After reverse transcription, the cDNA was diluted 1:3 and 3 μl were amplified by real-time PCR (StepOne™ System) in 20 μl using DyNAmy Flash Probe qPCR Kit (Thermo Scientific, Waltham, MA, USA) with conventional Applied Biosystems cycling parameters (40 cycles of 95 °C, 5 s, and 60 °C, 1 min). Primers for human and mouse $GABA_A$ receptor subunit α1 and β2 were obtained from Life Technologies (Waltham,

MA, USA). References of the primers are: GABRA1: Hs00971228_m1; GABRB2: Hs00241451_m1; gabra1: Mm00439046_m1; and gabrb2: Mm00433467_m1. After amplification, the size of the quantitative real-time PCR products was verified by electrophoresis on 2% (wt/vol) ethidium bromide-stained agarose gel. CDK4 was used as control housekeeping gene to assess the validity of the cDNA mixture and the PCR reaction. The gene expression of CDK4 was clearly detected in SH-SY5Y (data not shown), but not that of $GABA_A$ receptor.

2.11. Reactive oxygen species detection

Total level of mitochondrial reactive oxygen species (mtROS) and specific level of mitochondrial superoxide anion radicals were assessed using the fluorescent dye dihydrorhodamine 123 (DHR) and the Red Mitochondrial Superoxide Indicator (MitoSOX), respectively. SH-SY5Y cells were plated in 6 replicates into a black 96-well cell culture plate at a density of 1.5×10^4 cells/well. After neurosteroid treatment, cells were loaded with 10 μM of DHR for 15 min or 5 μM of MitoSOX for 90 min at room temperature in the dark on an orbital shaker. After washing twice with HBSS (Sigma), DHR, which is oxidized to cationic rhodamine 123 localized within the mitochondria, exhibits a green fluorescence that was detected using the multilabel plate reader VictorX5 at 485 nm (excitation)/538 nm (emission). MitoSOX, which is specifically oxidized by mitochondrial superoxide, exhibits a red fluorescence detected at 535 nm (excitation)/595 nm (emission). The intensity of fluorescence was proportional to mtROS levels or superoxide anion radicals in mitochondria.

2.12. MnSOD activity

The DetectX Superoxide Dismutase (SOD) Activity Kit (Ann Arbor, MI, USA) was used to quantitatively measure manganese SOD (MnSOD) activity following the instructions of the manufacturer. Briefly, 1×10^6 SH-SY5Y cells were collected for protein extraction. After a short sonication in PBS, the cellular homogenate was centrifuged at 1500 ×*g* for 10 min at 4 °C. The supernatant was then centrifuged at 10,000 ×*g* for 15 min and the obtained cell pellet was treated with 2 mM potassium cyanide, and assayed for MnSOD activity.

2.13. Mitochondrial redox environment

To investigate changes in mitochondrial redox environment, SH-SY5Y cells were transfected with a plasmid coding for a redox sensitive green fluorescent protein with a mitochondrial targeting sequence (pRA305 in pEGFP-N1). In an oxidized environment the absorption increases at short wavelengths (390 nm) at the expense of absorption at longer wavelengths (485 nm). The fluorescence ratio indicates oxidation/reduction, *i.e.*, the redox environment in the mitochondria [28]. Cells were plated in 6 replicates into a black 96-well cell culture plate at a density of 1.5×10^4 cells/well. After neurosteroid treatment, cells were washed twice with PBS and placed in a HEPES buffer (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM D-glucose, and 20 mM HEPES). The ratio 390 nm/485 nm was measured using the multilabel plate reader VictorX5 detecting fluorescence at 390 nm or 485 nm (excitation)/510 nm (emission). An increase of the ratio indicates a more oxidized environment.

2.14. Statistical analysis

Data are given as the mean ± SEM, normalized to the untreated control group (= 100%). Statistical analyses were performed using the Graph Pad Prism software. For statistical comparisons of more than two groups, One-way ANOVA was used, followed by Dunnett's multiple comparison test *versus* the control. For statistical comparisons of two groups, Student unpaired *t*-test was used. P values < 0.05 were considered statistically significant. Statistical correlations were determined using Pearson's correlation coefficients.

3. Results

3.1. Neurosteroids modulate mitochondrial bioenergetics

To investigate the effects of neurosteroids on cellular bioenergetic activity, we first studied the SH-SY5Y cell line, a commonly used neuronal culture *in vitro* model that expresses a variety of neuronal receptors, including steroid receptors (progesterone, estrogen and androgen receptors) [29,30]. Cells were treated with different neurosteroids: progesterone (P), estradiol (E2), estrone (E1), testosterone (T), 3α-androstanediol (3α-A), DHEA (D) or allopregnanolone (AP), at two physiologically relevant concentrations, 10 nM and 100 nM [31–35], and ATP level was measured after 24 h of treatment. All neurosteroids, except allopregnanolone, were able to significantly increase ATP level (Fig. 2A), ranging from a 10% increase after 3α-A treatment (10 nM) up to a 22% increase induced by progesterone (100 nM) compared to the untreated control.

A pre-treatment for 1 h with different steroid receptor antagonists (SRA) including the progesterone receptor antagonist RU-486 (assay concentration 100 nM), the estrogen receptor antagonist ICI-182.780 (assay concentration 100 nM), and the androgen receptor antagonist 2OH-flutamide (assay concentration 1 μM) completely abolished the action of P, E2 and E1, as well as T and 3α-A, respectively (Fig. 2B). The SRAs alone were devoid of the effects of ATP production. These results indicate that the action of neurosteroids may be mediated by nuclear receptors *via* gene regulation, at least for those neurosteroids that act *via* these receptors (progesterone, estrogens, and androgens).

To exclude that this rise in ATP was due to enhanced cell proliferation, we investigated the effects of the different neurosteroids. Of note, only allopregnanolone at 100 nM induced a significant increase of cell division by about 6% compared to the control (Table 1). Thus, our results indicate that the neurosteroid-induced up-regulation of cellular energy levels was independent of cell proliferation demands.

To verify whether the increase of ATP levels was directly linked to mitochondrial activity, we investigated the effects of neurosteroids on MMP, an indicator of the proton motive force necessary for ATP synthesis by the mitochondrial ATP synthase [36]. Our results show that, at least for one of the two concentrations tested, neurosteroids induced a significant increase in MMP (Fig. 2C). The low concentration of 10 nM was particularly effective, ranging from an 18% increase after estradiol treatment up to a 32% increase induced by DHEA. Again, allopregnanolone was not able to significantly modulate the MMP. Thus, the observed increase in ATP is consistent with the finding of a slight hyperpolarization of the MMP.

Because molecules of ATP are produced by two main pathways, the cellular glycolysis and oxidative phosphorylation (OXPHOS) in mitochondria, we determined whether and which of those neurosteroids were able to modulate one or both pathways. For this purpose, we simultaneously monitored in real-time the extracellular acidification rate (ECAR), an indicator of glycolysis, and the oxygen consumption rate (OCR), an indicator of basal respiration, using a Seahorse Bioscience XF24 Analyser (Fig. 3A–C). On the one hand, despite a slight general increase, only estradiol and DHEA were able to significantly modulate the ECAR after 24 h of treatment (about 16.4% and 19.4% respectively) (Fig. 3A). On the other hand, our findings demonstrate that estradiol, estrone, testosterone, 3α-A and DHEA significantly increased the OCR, with the most pronounced effect detected after a testosterone treatment at 100 nM (+26.5% compared to the control) (Fig. 3B). To compare the action of neurosteroids on glycolysis and basal respiration, we characterized the bioenergetic profile of SH-SY5Y neuroblastoma cells, representing OCR *versus* ECAR under the different treatment conditions (Fig. 3C). Notably, after treatment with the neurosteroid

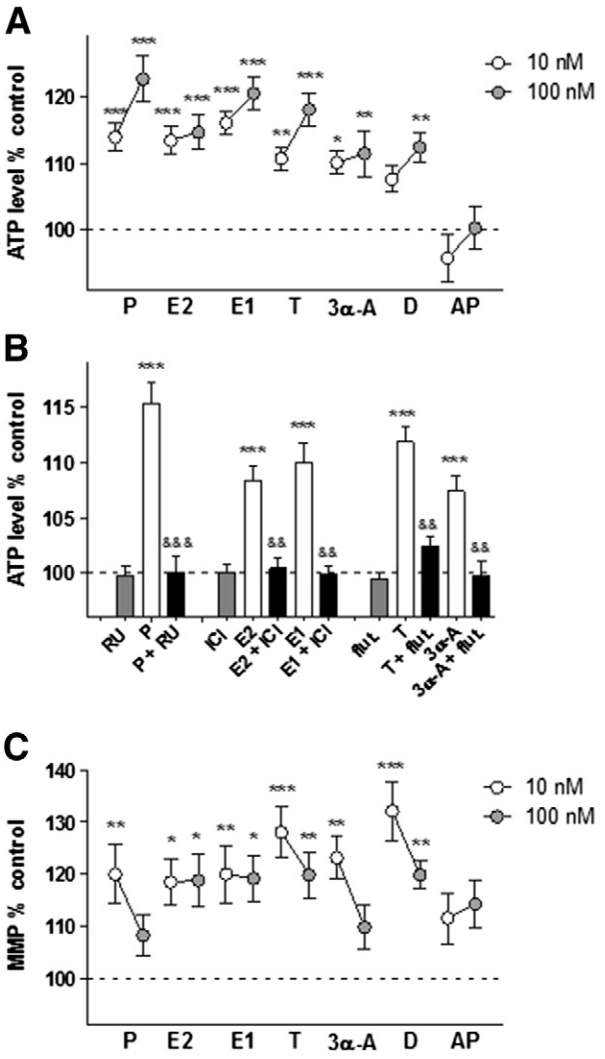

Fig. 2. Neurosteroids increase ATP level in SH-SY5Y neuroblastoma cells. (A) ATP level was significantly increased after neurosteroid treatment for 24 h at a concentration of 10 nM (white boxes) or 100 nM (gray boxes). (B) ATP level was measured after pre-treatment of cells for 1 h with either progesterone receptor antagonist RU-486 (100 nM), or estrogen receptor antagonist ICI-182.780 (100 nM), or androgen receptor antagonist 2OH-flutamide (1 μM) and then treated for 24 h with the corresponding steroid agonist. (C) Mitochondrial membrane potential (MMP) was significantly increased after neurosteroid treatment for 24 h at a concentration of 10 nM (white boxes) or 100 nM (gray boxes). (A–C) Values represent the mean ± SEM; n = 12–18 replicates of three independent experiments. One-way ANOVA and *post hoc* Dunnetts' multiple comparison test *versus* control (untreated), $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Student unpaired *t*-test, $^{\&\&}P < 0.01$; $^{\&\&\&}P < 0.001$. P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α-A; 3α-androstanediol, D; dihydroepiandrostanedione (DHEA), AP; allopregnanolone, RU; RU-486, ICI; ICI-182.780, flut.; 2OH-flutamide.

panel cells were switched to a metabolically more active state, with a tendency to increase both, glycolytic activity (ECAR) and basal respiration (OCR).

A Pearson correlation was performed to study whether the ATP levels correlated with OCR, ECAR or MMP (Fig. 4). A positive linear correlation between ATP level and OCR (Fig. 4A), but not between ATP and ECAR (Fig. 4B) or MMP (Fig. 4C) was detected, suggesting that the improvement in ATP production was preferentially linked to an increase of mitochondrial respiration (oxygen consumption).

To investigate more deeply the effects of neurosteroids on mitochondrial OXPHOS, OCR was measured using permeabilized SH-SY5Y cells, which allows the evaluation of different respiratory states and the respiratory control ratio (RCR = state 3/state 4) (Fig. 5). Especially testosterone significantly up-regulated the mitochondrial respiratory capacity by increasing the respiratory state 3 (ADP-dependent), state 3 uncoupled (in the absence of proton gradient after injection of FCCP) and the RCR (Fig. 5A–C). The treatment with DHEA (10 nM) showed a similar effect on the RCR under these experimental conditions (Table 1), while the rest of the tested steroid compounds had no beneficial effect on RCR. Thus, our findings suggest that neurosteroids primarily act on basal respiration in neuroblastoma cells, and that testosterone and DHEA are additionally able to increase the capacity for substrate oxidation (high RCR), which is important when cells have specific or high energy demands.

Since SH-SY5Y cells and other cell lines are not as highly dependent on OXPHOS as primary cell cultures to produce ATP [37], we investigated the action of neurosteroids on primary cell cultures from mouse brain cortex. Data demonstrate that, except for the treatment with progesterone, the level of ATP was significantly increased with at least one of the two concentrations tested, ranging from a 27% increase after treatment with estrone (100 nM) up to a 59% induced increase by DHEA (10 nM) (Fig. 6A). Compared to the data obtained with SH-SY5Y neuroblastoma cells (Fig. 2A), the magnitude of the rise in ATP concentration was higher in the primary cortical cell culture (maximal increase of 22.6% in SH-SH5Y cells *versus* 59.2% in primary neurons). This result implies that primary cell cultures have a greater capacity to produce ATP than neuroblastoma cells. Moreover, both concentrations of allopregnanolone were able to increase ATP level in primary cells, which was not the case in SH-SY5Y cells. Allopregnanolone mainly acts as an allosteric positive modulator of $GABA_A$ receptor ($GABA_A$-R). To verify the implication of this receptor, we first investigated whether it was expressed in both cell types. We found that SH-SY5Y cells do not express the $GABA_A$-R subunits α1 and β2 that are involved in the allopregnanolone binding site [38], in contrast to primary cortical neurons (Suppl. Fig. 1), indicating that $GABA_A$-R may be involved in the modulation of bioenergetics by allopregnanolone in neurons.

To determine whether the increase in ATP level was due to an improvement of glycolytic activity or mitochondrial respiration in this cellular model, we again performed a simultaneous real-time monitoring of the ECAR and the OCR (Fig. 6B–D). We measured a significant effect on the OCR for most of the neurosteroids tested, starting with a 59% increase after treatment with testosterone (10 nM) up to a 128% increase induced by 3α-A (10 nM) (Fig. 6B). Again, the magnitude of change was higher compared to the neuroblastoma cell line (maximal increase of 26.5%). In parallel, we measured a slight, but not significant, decrease in the glycolytic activity, except for the treatment with progesterone at 100 nM which in contrast induced a huge increase in the ECAR (+51.% compared to the control condition) (Fig. 6C). The bioenergetic profile (OCR *versus* ECAR) revealed that after treatment with neurosteroids, the primary cortical neurons had the general tendency to switch to a more aerobic state (Fig. 6D) by increasing their oxygen consumption (OCR) and decreasing their glycolytic activity (ECAR), especially at the low concentration of 10 nM.

Taken together, these data indicate that in primary mouse neurons, most of the neurosteroids from the tested panel were able to increase ATP production *via* improvement of mitochondrial respiration.

3.2. Neurosteroids modulate the redox homeostasis

The increase of OXPHOS is often coupled with an increase in mitochondrial reactive oxygen species (mtROS) production [39,40]. Since neurosteroids were able to significantly increase mitochondrial respiration, we investigated whether ROS levels were also increased within mitochondria (mtROS) by measuring the oxidation of the fluorescent dye dihydrorhodamine 123 (DHR). All neurosteroids induced a significant dose-dependent increase in mtROS levels, ranging from a 43% increase after DHEA treatment (10 nM) up to a 111.3% increase induced by testosterone (100 nM) (Fig. 7A). Moreover, the specific measure of mitochondrial superoxide anion radicals revealed that some of the ROS produced were indeed superoxide anions (Table 1). However, the extent of mtROS production, which in excess can lead to massive oxidative stress,

Table 1
Effects of neurosteroids on cellular bioenergetics in neuroblastoma cells.

		Progesterone		Estradiol		Estrone		Testosterone		3α-androstanediol		DHEA		Allo-pregnanolone	
		10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM
ATP level		113.9*	122.6*	113.4*	114.6*	116*	120.4*	110.7*	118*	110.1*	111.4*	107.6*	112.4*	95.8	100.3
Cell proliferation		98.7	100.9	98.2	100.3	97.2	99.2	97.9	102.1	100.1	103.8	97.6	103.7	101.7	106.2*
MMP		120*	108.2	118.5*	118.8*	120*	119.2*	128.1*	119.9*	123.2*	109.8	132.2*	119.9*	111.5	114.2
Glycolysis		115.4	102.7	119.4*	116.1*	113.8	111.1	108.1	105.5	105.7	106.6	106.2	116.4*	102.2	101.6
Mitochondrial respiration	Basal	111	105.9	118.3*	123.2*	110.9	118.1*	115.1*	126.5*	106.9	114.1*	110.8*	106.7	99.6	109.6
	RCR	97.9	98.7	93.7	89.6	100.6	102.8	132.7*	116.5*	95.3	104	120.3*	97.81	105.7	102.1
Mitochondrial ROS	Total	108.4	151.7*	122.9	160.8*	121.8	171.3*	145.2*	211.3*	148.4*	182.8*	143.9*	200.6*	151*	207.2*
	Superoxide	100.7	104*	103	106.2*	102.8	108*	103.6*	105.8*	103.8*	105.9*	105*	106.4*	103.6*	107.4*
MnSOD activity		110.6	128.6*	120.7	136.6*	128.7	141.9*	137.4*	149.3*	134.7*	127.6	135.3*	128.1*	128.7*	147.4*
Mitochondrial redox state		105.7	110.6	108.1	108.9	104.1	109.2	109.9	113.6*	109.8	109.1	104.2	113.3*	91.7	102.5

Values represent the mean normalized on 100% of the control group (untreated).
* Indicates when the percentage is significantly different from the control group. MMP; mitochondrial membrane potential, RCR; respiratory control ratio, ROS; reactive oxygen species, MnSOD; manganese superoxide dismutase.

and finally cell death, did not seem to be sufficient to trigger cell death under those experimental conditions (data not shown).

Therefore, we next tested the antioxidant defense system in mitochondria. We quantitatively measured the activity of the manganese superoxide dismutase activity (MnSOD), which is present within the mitochondrial matrix. Indeed, MnSOD activity was significantly increased (Fig. 7B) after treatment with the whole panel of neurosteroids, ranging from a 28.6% (progesterone, 100 nM) up to a 49.3% increase (testosterone, 100 nM). The increase in mtROS was paralleled by an increase of antioxidant activity. In addition, mtROS level and MnSOD activity correlated with one another (Fig. 7C), suggesting that the increase of MnSOD activity was substrate-dependent.

Finally, to verify whether the mitochondrial redox environment was impacted by this increase of ROS *versus* increase of antioxidant defenses, SH-SY5Y cells stably transfected with a reporter gene coding for a redox sensitive green fluorescent protein (AR305 roGFP) located within mitochondria were treated with our panel of neurosteroids [28]. Fig. 7D displays the oxidation/reduction state in mitochondria, and indicates that, despite a slight switch toward a more oxidized state, only testosterone (100 nM) and DHEA (100 nM) significantly modified the redox environment in mitochondria.

Taken together, our data indicate that neurosteroids increased mitochondrial activity, which was paralleled by an enhancement in mtROS levels. However, cell viability was still unchanged and the raise of mtROS appeared to be at least in part compensated by an increase in antioxidant activity, which in turn led to a slight switch to an oxidized state within mitochondria.

4. Discussion

The aim of our study was to investigate the effects of seven neurosteroids on cellular bioenergetics and redox homeostasis in neuronal cells. The key findings were that: i) the majority of these steroids increased energy metabolism, mainly *via* an up-regulation of the mitochondrial activity and at least in part *via* receptor activation, and ii) neurosteroids regulated redox homeostasis by increasing the antioxidant activity as a compensatory mechanism to the ROS level enhancement which might result from the acceleration in oxygen consumption accompanied by a greater electron leakage from the electron transport chain. Additionally, each neurosteroid seems to have a specific bioenergetic profile. The single profiles are delineated as pie charts for SH-SY5Y (Fig. 8A) and mouse primary cortical neurons (Fig. 8B).

Remarkably, each steroid doesn't seem to act in the same way on both cell types. For example, allopregnanolone, which had no effects on ATP level and basal respiration in SH-SY5Y cells, appeared to increase those two parameters in primary neuronal cells. On the contrary, progesterone was able to increase ATP production in SH-SY5Y cells, but showed a significant effect only on glycolysis in primary cells. One explanation could be that SH-SY5Y cells and primary neuronal culture may exhibit steroid receptor expression profiles that are slightly different. Steroid receptor expression, such as that of progesterone, estrogen and androgen receptors, has already been demonstrated in both SH-SY5Y cells [29,30] and in mouse neurons [41–43], respectively. It is known that allopregnanolone doesn't bind to a conventional steroid receptor but mainly acts as a positive $GABA_A$ receptor ($GABA_A$-R) allosteric modulator that strengthens the effects of GABA. We found that SH-SY5Y cells do not express $GABA_A$-R unlike in the case of primary neurons (Suppl. Fig. 1). This indicates that allopregnanolone may act *via* $GABA_A$-R to increase ATP level especially in primary neurons and explains the lack of effect on ATP in SH-SY5Y cells. Furthermore, other signaling pathways and receptors may be involved in the effects of allopregnanolone upon bioenergetics in primary cortical neurons, such as the newly characterized pregnane xenobiotic receptor [44].

Moreover, it is known that proliferative cells and tumors have a net tendency to use the cellular glycolysis to produce ATP instead of the OXPHOS system. This phenomenon is called “Warburg effect” [37]. On the contrary, primary neurons, which are differentiated cells, rely almost exclusively on the OXPHOS system to produce ATP and glycolysis is really low (raw data not shown). Indeed, in the latter model, ATP level appeared to be strictly coupled with the basal respiration. The bioenergetic profile of primary cortical cells revealed that neurosteroids preferentially increased mitochondrial respiration and not the glycolytic pathway, while both pathways were increased in SH-SY5Ycells (Figs. 3C, 6D).

In the recent years, neurosteroids have emerged as new potential therapeutic tools against neurodegeneration [45]. Among the steroids, the family of sex steroid hormones is the most widely studied. They are in the focus of research on neurodegenerative diseases since cognitive decline and the risk to develop AD appear to be associated with an age-related loss of sex hormones (*e.g.* estradiol, testosterone but also progesterone) in both, women and men [25,46], a hypothesis largely supported by epidemiological evidence [47]. *In vitro* and *in vivo* studies demonstrated neuroprotective effects of sex hormones, particularly with mitochondria proposed as the primary site of action of estradiol [24,32,48,49]. Indeed, estrone (E1), estradiol (E2), and estriol (E3), are known to play a fundamental role in the regulation of the female metabolic system [50]. It has been reported that estrogens can regulate mitochondrial metabolism by increasing the expression of glucose transporter subunits and by regulating some enzymes involved in the tricarboxylic acid cycle (TCA cycle) and glycolysis, which leads to an improvement in glucose utilization by cells (reviewed in [21]). Estrogens seem also able to up-regulate genes coding for some electron transport chain components such as subunits of mitochondrial complex I (CI), cytochrome *c* oxidase (complex IV), and the F1 subunit of ATP synthase. In line with these findings, our data demonstrated that both female sex hormones, estradiol (E2) and estrone (E1), were able to increase ATP

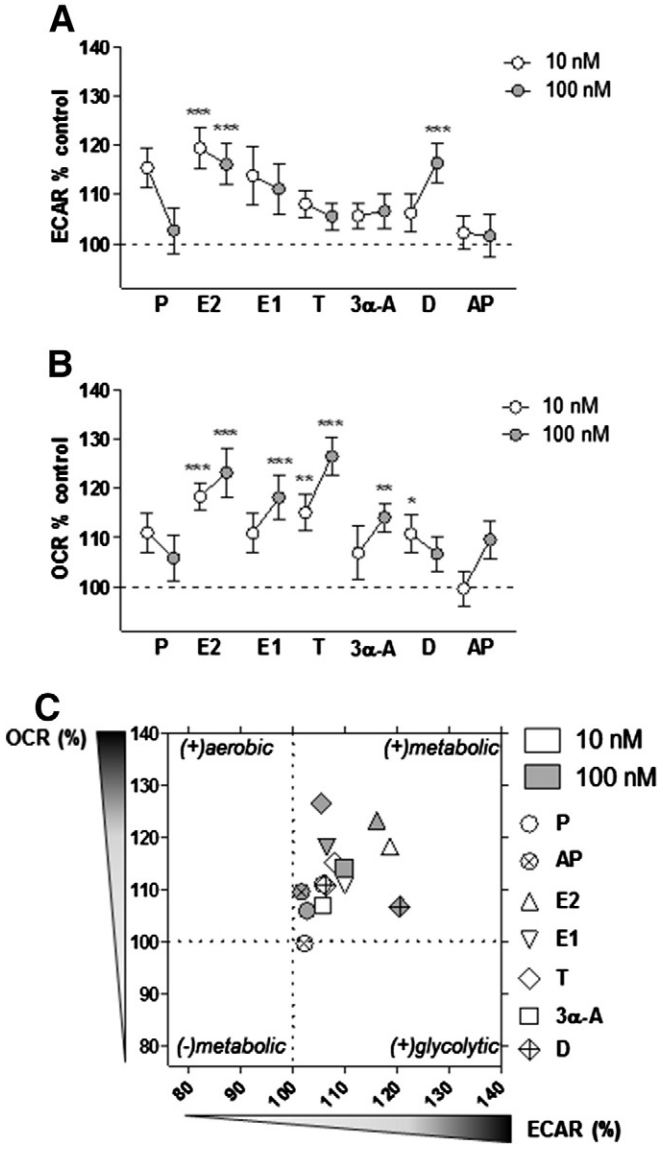

Fig. 3. Neurosteroids positively regulate bioenergetic activity in SH-SY5Y neuroblastoma cells. (A) Extracellular acidification rate (ECAR) and (B) oxygen consumption rate (OCR) were measured simultaneously using a Seahorse Biosciences XF24 Analyser in the same experimental conditions. (C) Bioenergetic profiling of SH-SY5Y cells (OCR *versus* ECAR) revealed increased metabolic activity after treatment with neurosteroids. Values represent the mean of each group (mean of the ECAR in abscissa/mean of the OCR in ordinate) normalized to the untreated control group (=100%). (A–C) Values represent the mean ± SEM; n = 12–18 replicates of three independent experiments. One-way ANOVA and *post hoc* Dunnetts' multiple comparison test *versus* control (untreated), *P < 0.05; **P < 0.01; ***P < 0.001; P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α-A; 3α-androstanediol, D; dihydroepiandrostanedione (DHEA), AP; allopregnanolone.

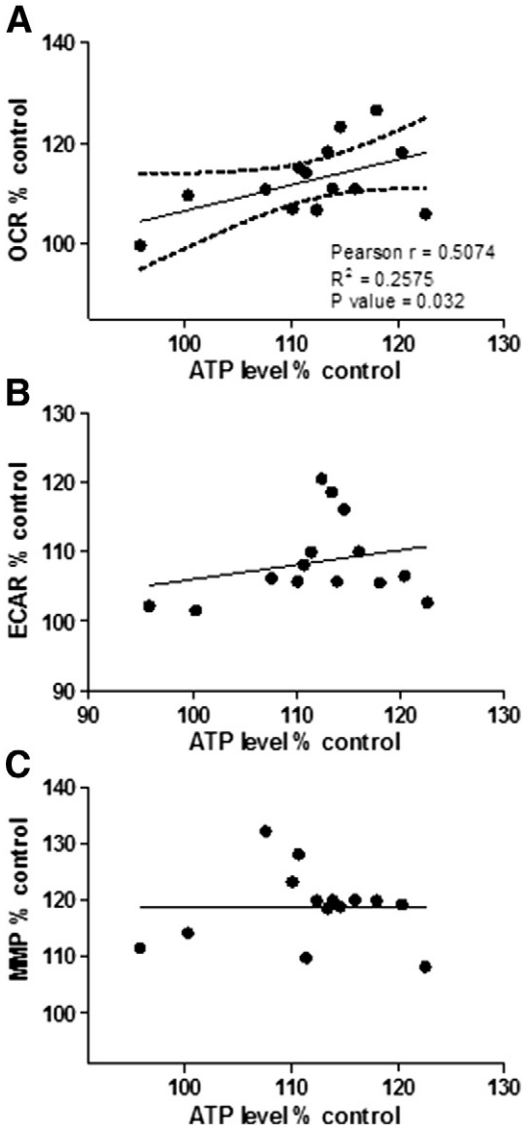

Fig. 4. ATP levels did correlate with basal mitochondrial respiration. Graph representing ATP levels in abscissa *versus* (A) OCR or (B) ECAR or (C) MMP in ordinate. Values represent the mean of each treatment group normalized to the control group (=100%). Pearson correlation r = 0.5074, R^2 = 0.2575, P = 0.0032. OCR; oxygen consumption rate, ECAR; extracellular acidification rate; MMP; mitochondrial membrane potential.

levels, basal respiration, and MMP in neuroblastoma cells (Fig. 8A). Of note, the increase of ATP levels induced by E2 and E1 was abolished in the presence of ICI-182.780, an estrogen receptor (α and β) antagonist (Fig. 2B) suggesting that estrogens, such as E2 and E1, may act *via* these receptors to up-regulate genes involved in cellular bioenergetics, as mentioned above. Estradiol seemed to be more potent that estrone, because both concentrations (10 nM and 100 nM) were effective to increase ATP levels and mitochondrial respiration. In addition, estradiol was able to regulate glycolysis. This difference can be explained by the observation that, despite estrone's capability as an estrogenic compound, it is about 10 times less estrogenic than estradiol [21]. The same finding was observed in primary neurons (Fig. 8B).

Regarding the predominantly male hormone testosterone, we witnessed an increase in ATP levels, basal respiration and mitochondrial membrane potential in neuroblastoma cells (Fig. 8A). Moreover, testosterone was also the only steroid besides DHEA inducing an acceleration of the respiratory control ratio (RCR), an indicator of the capacity for substrate oxidation (high RCR), which is important when cells have specific or high energy demands. The role of androgens on mitochondrial function, especially testosterone, has received little attention up to now, compared to the estrogens. Only one study demonstrated a similar effect of testosterone on MMP [51]. Furthermore, it has been proposed that estradiol and testosterone can regulate energy production by inducing nuclear and mitochondrial OXPHOS genes, since the subunits of mitochondrial chain complexes are encoded by the nuclear and the mitochondrial genome, respectively, and both contain hormone responsive elements [52]. Again, those findings are in line with our results, since we have shown that the increase of ATP levels was blocked in the presence of estrogen and androgen receptor antagonists (Fig. 2B).

Progesterone is the second main female sex hormone but it is also a precursor for estrogens and androgens. Progesterone, and its 3α–5α-derivate allopregnanolone (or 3α, 5α-tetrahydroprogesterone) as well as 3α-androstanediol, seem to play a role in mood modulation. Their

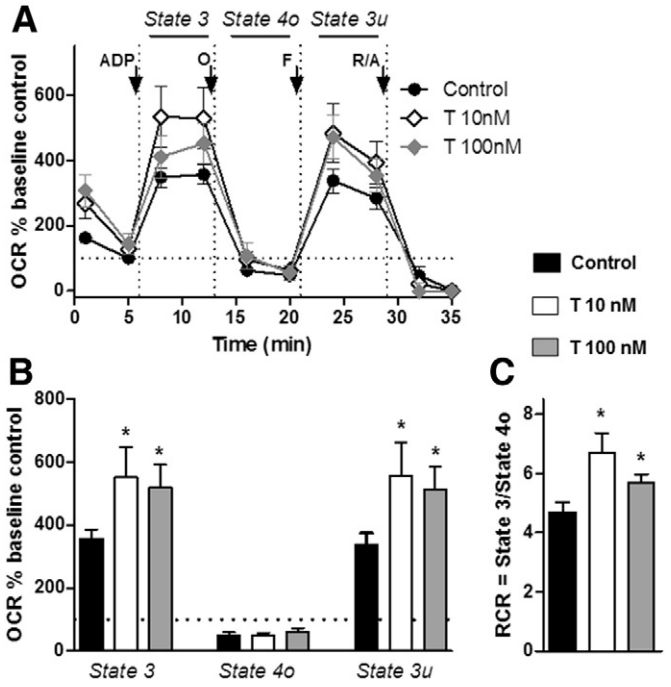

Fig. 5. Testosterone increased mitochondrial respiratory capacity. (A) Oxygen consumption rate (OCR), was measured on permeabilized SH-SY5Y cells after treatment with testosterone for 24 h, using a XF24 Analyser (Seahorse Bioscience). The sequential injection of mitochondrial inhibitors is indicated by arrows (see details in the Materials and methods section). (B) Values corresponding to the different respiratory states are represented as mean ± SEM (n = 15–18 replicate of three independent experiments/group) and were normalized to the basal respiration of the control group (=100%). (C) The respiratory control ratio (RCR = State 3/State 4o), which reflects the mitochondrial respiratory capacity, was increased by testosterone. Student unpaired *t*-test, *P < 0.05. T 10 nM; testosterone at a concentration of 10 nM, T 100 nM; testosterone at a concentration of 100 nM, O; oligomycin, F; FCCP, R/A; rotenone/antimycin A.

therapeutic potential for the treatment of depression, anxiety [53–55] and more recently AD is currently under investigation [35]. In the present study, we demonstrated that progesterone increased ATP levels and MMP without significant effects on basal respiration in neuroblastoma cells (Fig. 8A). An increase in glycolysis was also observed after treatment in the primary neurons (Fig. 8B). Again, the up-regulatory effect of progesterone on ATP levels was shut down in the presence of the progesterone receptor antagonist RU-486 (Fig. 2B), suggesting that progesterone also modulates cellular bioenergetics by regulating gene expression *via* a progesterone nuclear receptor. Studies performed on ovariectomized rats revealed that a 24 h treatment with progesterone (subcutaneous injection, 30 μg/kg) increased OXPHOS capacity in isolated mitochondria, in part by enhancing cytochrome *c* oxidase activity and expression [32]. Interestingly, the increase of OXPHOS capacity was suppressed by a co-treatment with estradiol and progesterone, suggesting a competitive mode of action between both steroids. Another study using wobbler ALS (amyotrophic lateral sclerosis) model mice showed that progesterone was able to normalize the deficits in mitochondrial complex I activity observed in motor neurons of the cervical spinal cord [56]. Because progesterone seems to have different functional effects, one can speculate that its action on mitochondrial respiration may be distinct to specific nerve cell populations.

Allopregnanolone and 3α-androstanediol have a distinct mode of action compared to sex hormones because they mainly act on membrane receptors (allosteric modulator of $GABA_A$-R) and not nuclear receptors [57]. Their effects on mitochondrial bioenergetics cannot be explained by a direct regulation of genes involved in the OXPHOS system as previously proposed for sex hormones. In our study, 3α-androstanediol showed a similar effect compared to progesterone in the neuronal cell line, but was also able to significantly increase the basal respiration (at 100 nM) (Fig. 8A). Both concentrations were effective to increase ATP and respiration in primary cells (Fig. 8B). Allopregnanolone significantly regulated ATP levels and basal respiration only in primary neurons, whereas no effect was detected in the neuroblastoma cell line. Based on those observations, we can speculate that: i) $GABA_A$-R is involved in the up-regulatory effect of allopregnanolone on ATP levels in primary cells because no increase was observed in SH-SY5Y cells lacking of this receptor (Suppl. Fig. 1); and ii) 3α-androstanediol could act *via* androgen receptor because its effect on ATP levels was abolished in the presence of an androgen receptor antagonist (Fig. 2B). However, further investigations will be required to understand the exact underlying mechanisms. Besides, due to the high complexity of the neurosteroid pathway synthesis, it is difficult to conclude that the effect which we observed on bioenergetics is due to the tested neurosteroid itself, or to one of its metabolites, because they all belong to crisscross pathways (Fig. 1). However, since blocking progesterone, estrogen and androgen receptors abolishes the effects of their respective agonists, we have good evidence that the neurosteroids themselves exhibit the mode of action. In the same way, we can exclude that progesterone is acting *via* its metabolite allopregnanolone because the latter has no effects on bioenergetics in SH-SY5Y cells. Nevertheless, it is also possible that 3α-androstanediol doesn't act directly on androgen receptors but is converted in dihydrotestosterone, another testosterone metabolite which has high affinity for this receptor. In a similar way, DHEA can be converted in androgens and estrogens and may act *via* the corresponding steroid nuclear receptor.

DHEA (dehydroepiandrosterone) was the first neurosteroid identified in 1981 [11], and its physiological action involves both genomic and non-genomic mechanisms, in part *via* activation of androgen/estrogen receptors and allosteric modulation of NMDA receptors, respectively [58]. Human studies showed an age-related decrease in DHEA levels in the brain and in the blood in relation to the age-associated cognitive decline [17,59]. *In vitro*, we showed that DHEA enhanced ATP levels and basal respiration in primary neurons (Fig. 8B). A similar effect was observed in the neuronal cell line with an additional increase in MMP, glycolysis and RCR (Fig. 8A, Table 1). In agreement with our findings, DHEA was able to improve mitochondrial respiration in the brain of old rats (18–24 months) which exhibited a decline in mitochondrial function when compared to young rats (8–10 weeks) [60]. More specifically, DHEA stimulated the respiratory state 3 in old rats which consequently was similar to that of untreated young rats. Furthermore, DHEA increased cytochrome *c* content in young and old mouse brains and enhanced mitochondrial dehydrogenase activities.

Thus, the different bioenergetic profiles we observed after treatment with our panel of steroids could be explained by their distinct abilities to directly or indirectly regulate the transcription of genes involved in glycolysis and oxidative phosphorylation (probably, *via* steroid nuclear receptors), but also the content and activity of mitochondrial respiratory complexes. Further investigations are required to determine in more detail which genes are involved in these processes.

Mitochondria are known to be paradoxical organelles. They can be compared to a double-edged sword that, on one hand, produces the energy necessary for cell survival, and on the other hand, induces the formation of ROS that can be harmful for cells when produced in excess with mitochondria as the first target of toxicity [39,40]. In our study, the increase in ATP appeared to be coupled to an increase in MMP and improved basal respiration (Fig. 8A). In parallel, we detected higher mitochondrial ROS levels, supporting the hypothesis that increased mitochondrial activity generates more ROS. The only exception was observed after treatment with allopregnanolone where we detected more ROS but no increase in ATP level, MMP, or basal respiration. We can speculate that, in this model, allopregnanolone might be able to increase ROS-producing metabolic functions *via* other mechanisms. But with regard to the other neurosteroids, the increase of mitochondrial ROS was paralleled by an increase in MnSOD activity. The MnSOD is located in the mitochondrial matrix and represents one of the first

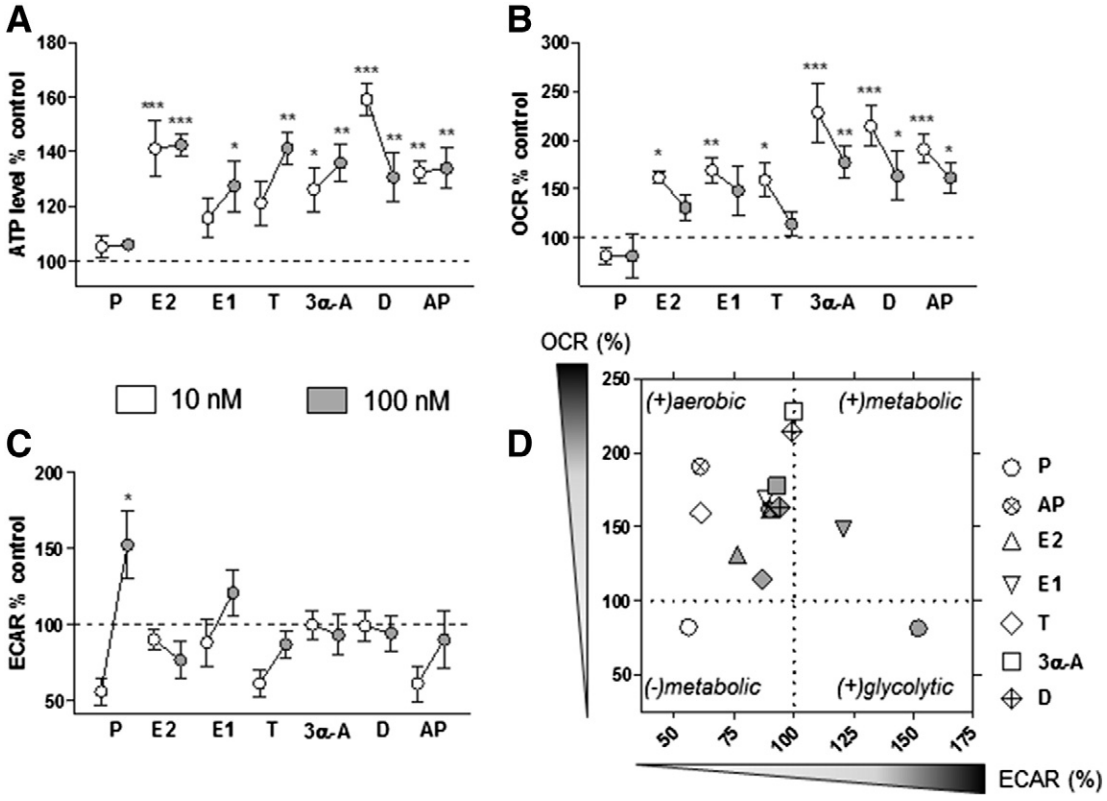

Fig. 6. Neurosteroids up-regulated the bioenergetic activity in primary cortical cells. (A) ATP level was significantly increased after neurosteroid treatment (24 h) at a concentration of 10 nM (white boxes) and 100 nM (gray boxes). (B) Oxygen consumption rate (OCR) and (C) extracellular acidification rate (ECAR) were measured simultaneously using a Seahorse Biosciences XF24 Analyser under the same experimental conditions. (D) Bioenergetic profile of primary cortical cells (OCR *versus* ECAR) revealed an increased aerobic activity (O_2 consumption) after treatment with neurosteroids. Values represent the mean of each group (mean of the ECAR in abscissa/mean of the OCR in ordinate) and were normalized to the control group (100%). (A–C) Values represent the mean ± SEM, n = 4–6 replicates of three independent experiments/group, and were normalized to the control group (=100%). One-way ANOVA and *post hoc* Dunnetts' multiple comparison test *versus* control (untreated), *P < 0.05; **P < 0.01; ***P < 0.001; P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α-A; 3α-androstanediol, D; dihydroepiandrostanedione (DHEA), AP; allopregnanolone.

antioxidant defenses against ROS produced by OXPHOS [61]. Its improved activity could be in part explained by an up-regulation of gene expression and protein level of MnSOD. Indeed, in studies which focused on antioxidant effects of steroids in ovariectomized female rats, an increase of MnSOD protein level has been observed after treatment with estradiol or progesterone [32], whereas DHEA preferentially up-regulated the expression of Cu/ZnSOD [31]. In orchiectomized male rats, testosterone was also able to increase MnSOD protein level compared to the control (sham operated) [62]. A similar observation was made in the wobbler ALS mouse model, where MnSOD expression was elevated after treatment with progesterone [56].

In our study, the correlation between mitochondrial ROS level and MnSOD activity implies that the increase of enzymatic SOD activity might be preferentially substrate-dependent, but can be explained, at least in part, by an up-regulation of gene expression.

Based on our observation, one can speculate that pre-treatment with neurosteroids may exert a protective action against oxidative stress, possibly through a preconditioning mechanism *via* their ability to increase antioxidant defenses (*i.e.* MnSOD activity). However, in an already oxidized environment, the presence of neurosteroids may be deleterious for cells because they also appear to further increase ROS production. This observation reinforces the "critical window hypothesis" of the therapeutic use of steroids as debated recently with regard to the hormone replacement therapy in post-menopausal women [63] and implies that this kind of therapy should begin at an age when the redox system is still balanced, thus favoring the reference postulating early onset administration.

It is known that some neurosteroid levels decline during aging and are further modified in neurodegenerative conditions (*i.e.* AD and PD). In addition, mitochondrial dysfunction has been well-documented in aging and age-related neurodegenerative diseases [64]. Steroids offer interesting therapeutic opportunities for promoting successful aging because of their pleiotropic effects in the nervous system. Our findings highlight, for the first time, up-regulatory effects of neurosteroids upon the neuronal bioenergetic activity *via* up-regulation of the mitochondrial oxygen consumption as a common mechanism underlying neurosteroid action. In addition, these steroids can modulate the redox homeostasis, by balancing the increase of ROS production *via* improved mitochondrial antioxidant activity (Fig. 9). Thus, our results provide new insights in re-defining the biological model of how neurosteroids control neuronal functions. Because each steroid appeared to have a specific profile in bioenergetic outcome and redox homeostasis, the underlying mechanisms have to be elucidated in more details in the future, as well as those in models of neurodegenerative diseases, such as AD.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.09.013.

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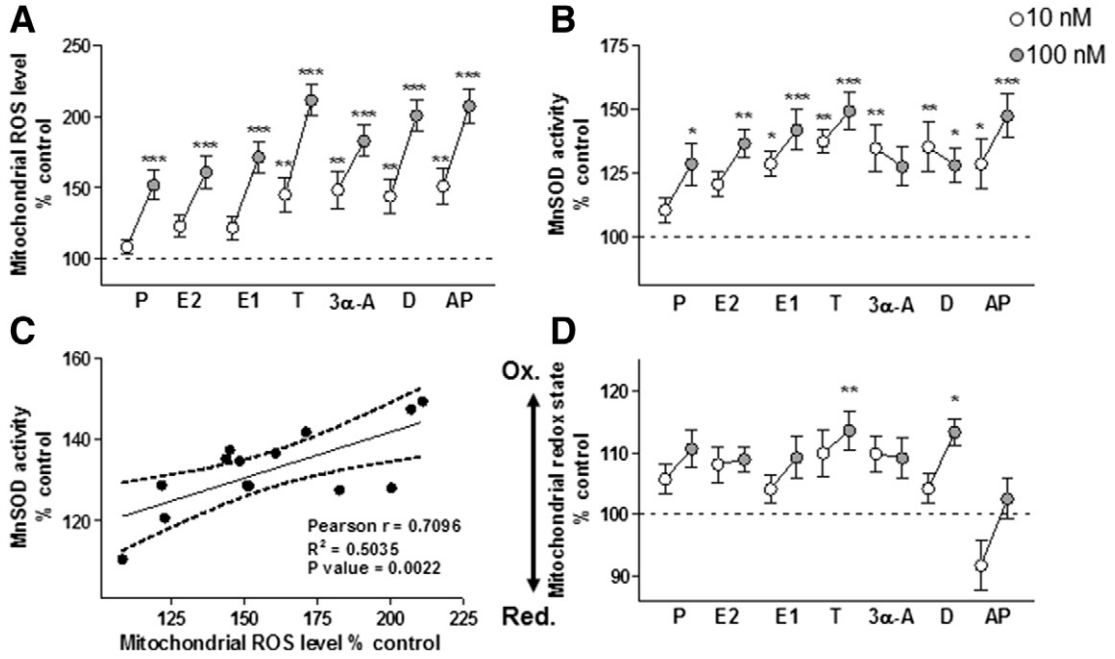

Fig. 7. Neurosteroids modulate the mitochondrial redox environment in SH-SY5Y neuroblastoma cells. (A) Mitochondrial reactive oxygen species (mtROS) levels were significantly increased after neurosteroid treatment (24 h) at a concentration of 10 nM (white boxes) and 100 nM (gray boxes). (B) This increase was accompanied by an up-regulation of manganese superoxide dismutase activity (MnSOD). (C) A positive correlation was observed between ROS levels and MnSOD activity. (D) Using a reporter gene coding for a redox sensitive green fluorescent protein (AR305 roGFP) located within mitochondria, the mitochondrial redox state underwent a switch to a more oxidized state after neurosteroid treatment compared to the untreated control. (A, B) Values represent the mean ± SEM and were normalized to the corresponding untreated control group (=100%). (C) Values represent the mean of each group (mean of the mitochondrial ROS level in abscissa/mean of the MnSOD activity in ordinate) normalized to the untreated control group (=100%). Pearson correlation r = 0.7096, R^2 = 0.5035, P = 0.0022. (D) Values represent the mean ± SEM of the ratio "oxidized state/reduced state", n = 8–15 replicates of three independent experiments/group. Values were normalized to the control group (=100%). One-way ANOVA and *post hoc* Dunnett's multiple comparison test *versus* control (untreated), *P < 0.05; **P < 0.01; ***P < 0.001; P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3a-A; 3a-androstanediol, D; dihydroepiandrostanedione (DHEA), AP; allopregnanolone, Ox.; oxidized environment, Red.; reduced environment.

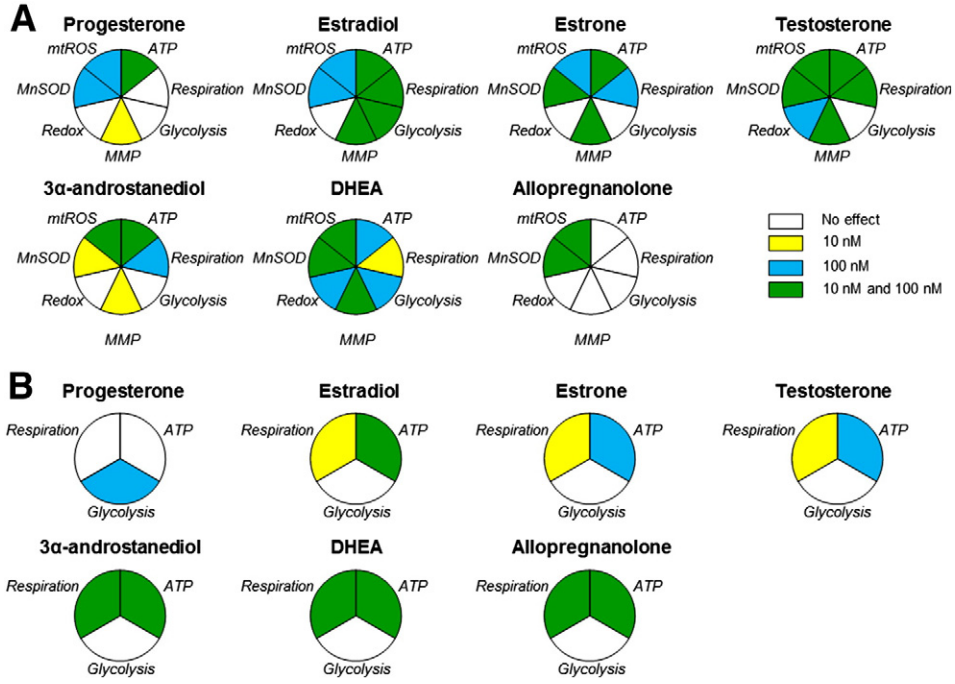

Fig. 8. Different action profile of neurosteroids on cellular bioenergetics. Representative diagrams of the effects of neurosteroids on the bioenergetic activity (ATP level, basal respiration, glycolysis, MMP) and the modulation of mitochondrial redox environment (mtROS levels, MnSOD activity, redox state) in SH-SH5Y neuroblastoma cells (A), and the bioenergetic activity in primary cortical cells (B). No effect is represented in white color. A significant increase of the respective parameter is marked either in yellow (significant only at 10 nM), blue (significant only at 100 nM), or green (significant at both concentrations). mtROS; mitochondrial reactive oxygen species, MMP; mitochondrial membrane potential, MnSOD; manganese superoxide dismutase activity.

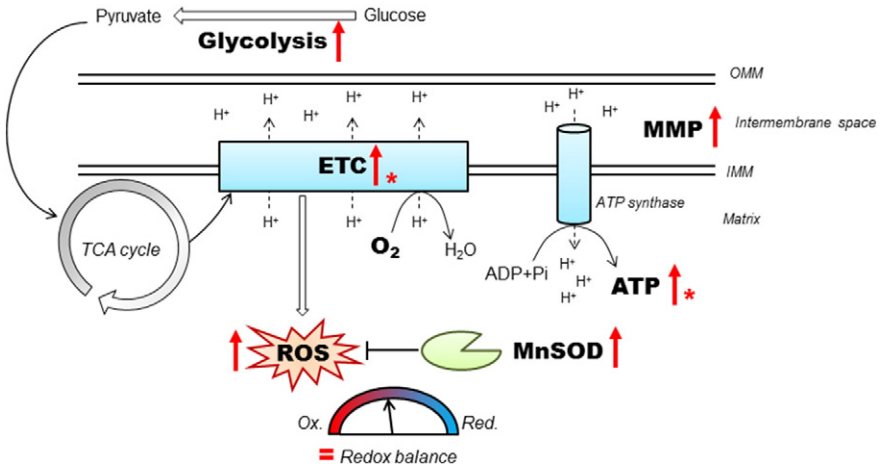

Fig. 9. Schematic representation of the effects of neurosteroids on mitochondrial bioenergetics and redox environment in SH-SH5Y neuroblastoma cells. * indicates that the effect was similar to that observed in primary cortical cells. ETC; electron transport chain, TCA; tricyclic acid, MnSOD; manganese superoxide dismutase, ROS; reactive oxygen species, MMP; mitochondrial membrane potential.

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